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LSD1 interacts with cMYB to demethylate repressive histone marks and maintains inner ear progenitor identity

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Summary Statement

The histone demethylase Lsd1 is required for inner ear progenitor specification and acts in a complex with the Myb transcription factor. Myb recruits Lsd1 to the promoter of key otic determinants, where it controls H3K9 demethylation to maintain their active transcription.

Abstract

During development, multipotent progenitor cells must maintain their identity while retaining the competence to respond to new signalling cues that drive cell fate decisions. This depends on both DNA-bound transcription factors and surrounding histone modifications. Here we identify the histone demethylase Lsd1 as a crucial component of the molecular machinery that preserves progenitor identity in the developing ear prior to lineage commitment. While Lsd1 is mainly associated with repressive complexes, we show that in ear precursors it is required to maintain active transcription of otic genes. We reveal a novel interaction between Lsd1 and the transcription factor cMyb, which in turn recruits Lsd1 to the promoters of key ear transcription factors. Here, Lsd1 prevents the accumulation of repressive H3K9me2 while allowing H3K9 acetylation. Loss of Lsd1 function causes rapid silencing of active promoters, loss of ear progenitor genes and shuts down the entire ear developmental programme. Our data suggest that Lsd1-cMyb acts as a coactivator complex that maintains a regulatory module at the top of the inner ear gene network.

Introduction

In the developing embryo, multipotent progenitors are able to maintain their identity and gene expression programmes despite ongoing changes in their signalling environment. We have recently uncovered a gene network that integrates signalling inputs and transcriptional activity during the transition from progenitor to committed inner ear cells (Anwar et al, 2017; Chen et al, 2017), providing a good basis to explore this problem in a well-defined system. The entire inner ear is derived from a simple epithelium, the otic placode, located in the ectoderm next to the hindbrain. Prior to placode formation, otic precursors are part of a sensory progenitor pool (the pre-placodal region, PPR), from which FGF signalling induces otic-epibranchial progenitors (OEPs) (Anwar et al, 2017; Ladher et al, 2000; Maroon et al, 2002; Martin & Groves, 2006; Sun et al, 2007; Urness et al, 2010; Wright & Mansour, 2003). Over time, OEPs segregate into committed ear and epibranchial cells. They do this gradually by responding to new signalling cues and by altering their transcriptional machinery (Chen et al, 2017; Freter et al, 2008; Jayasena et al, 2008; Park & Saint-Jeannet, 2008; Sun et al, 2007). Thus, during this transitional period OEPs must maintain their identity before proceeding to lineage commitment. The molecular mechanisms that maintain the competence of OEPs to respond to signals and maintain their progenitor state have not been identified.

Epigenetic mechanisms such as histone modifications are known to maintain progenitor cell identity as well as prime them for differentiation (Martin & Zhang, 2005), and may therefore play a role in keeping cells in an OEP state. One such modification is histone methylation in gene bodies and regulatory regions (Layman & Zuo, 2014; Ooi & Wood, 2007) associated with either gene activation or repression. In placode-derived sense organs, virtually nothing is known about the epigenetic control of cell identity as cells transit from progenitor to lineage commitment. The histone demethylase Kdm4b (also known as JmjD2B), which specifically removes di- and trimethyl groups from lysine 9 of histone 3

(H3K9me2/3) (Labbé et al, 2014), is the only epigenetic enzyme identified in inner ear precursors to date and controls placode invagination at later stages (Uribe et al, 2015). Recent RNAseq analysis (Chen et al, 2017) identified the demethylase Lsd1 as specifically enriched in OEPs, suggesting that it could be involved in ear specification.

Lsd1 plays a key role in maintaining the pluripotency of stem cells (Adamo et al, 2011), as well as acting as a molecular toggle switch that promotes or inhibits differentiation of progenitor cells (Laurent et al, 2015). Lsd1 (also known as Kdm1a/Aof2/BHC110) is a flavin-dependent demethylase that specifically removes active H3K4me1/2 or repressive H3K9me1/2 marks, and thus can function as a transcriptional repressor or activator (Forneris et al, 2006; Metzger et al, 2016; Metzger et al, 2005). Consistent with this, it is found in both corepressor and coactivator complexes such as REST-CoREST (Ballas et al, 2005; Lee et al, 2005; Shi et al, 2005) and androgen receptor (AR)-Chd1 complexes (Metzger et al, 2016; Metzger et al, 2005). Here, we show that Lsd1 is indispensable for ear development. It is required to maintain the expression of key progenitor-specific genes (*Sox8*, *Pax2*, *Etv4* and *Zbtb16*) that feed into a larger gene regulatory network that controls ear commitment. We identify cMyb as a novel Lsd1 interacting partner that recruits Lsd1 to promoter regions of actively transcribed genes to maintain the active histone mark H3K9ac by catalysing H3K9 demethylation.

Results

Lsd1 is expressed in OEPs after their specification

The commitment of OEPs towards an otic fate is a gradual process that requires the integration and fine-tuning of gene regulatory networks (Chen et al, 2017). OEP specification by FGF signals begins around somite stage (ss) 1 in chick embryos (Groves & Bronner-Fraser, 2000). By ss5 OEPs express high levels of the FGF mediators *Etv4* and *Etv5* (Anwar et al, 2017; Betancur et al, 2011; Lunn et al, 2007) and develop along the otic lineage when cultured in isolation (Groves & Bronner-Fraser, 2000). However, it is not until ss10 that the otic lineage becomes molecularly distinct from the epibranchial cells (Chen et al, 2017), becoming fully committed to otic fate by ss11-12 (Groves & Bronner-Fraser, 2000). This progressive commitment is driven by a sequential activation of different signalling pathways (Freter et al, 2008; Jayasena et al, 2008; Park & Saint-Jeannet, 2008; Shida et al, 2015). The mechanisms whereby OEPs maintain their identity and remain poised for rapid response to new signals have not been identified.

We hypothesised that epigenetic mechanisms may be involved, and using our recent RNA-seq data (Chen et al, 2017), we identified *Lsd1* as a candidate for maintaining OEP identity prior to lineage commitment. *Lsd1* is expressed immediately after OEP specification but prior to molecular segregation of the otic-epibranchial lineages. We confirmed this by *in situ* hybridisation. At ss5, *Lsd1* is confined to the neural tube, with weak expression in the mesoderm, but absent from OEPs (Fig.1A, a). At ss8, it becomes expressed in OEPs (Fig.1B, b), and by ss13, it is robustly expressed in the otic placode and weakly in more lateral ectoderm (Fig.1C, c). To determine the onset of *Lsd1* expression, we performed qPCR from dissected OEPs and placodes, and compared its expression to that of the transcription factor *Pax2,* an established, FGF-responsive OEP marker (Chen et al, 2017; Nechiporuk et al, 2007; Phillips et al, 2001; Yang et al, 2013). This analysis confirms the absence of *Lsd1* from OEPs at ss5 and its upregulation at ss8 (Fig.1D), preceding the

segregation of otic and epibranchial lineages. Thus, *Lsd1* expression is activated in OEPs after they have been specified, and its expression persists as otic cells become committed.

Lsd1 is necessary for otic placode formation and subsequent differentiation

To test directly if *Lsd1* is required to maintain OEP identity, we performed knock-down experiments using two different morpholinos (MO) against *Lsd1* (Lsd1-MO; both affect *Lsd1* splicing; Fig.S1A). We find that Lsd1 knock-down with either MO leads to a dramatic reduction of otic vesicle size as compared to the control side (n=16, Fig.2A-b') indicating an important role for Lsd1 in ear development.

To understand global changes in the otic gene network after Lsd1 knockdown, we designed a NanoString probeset containing 220 transcripts based on our RNAseq data (Chen et al, 2017), which includes 70 otic genes as well as factors specific for other placodes, neural, epidermal and neural crest cells (TableS1). Lsd1-MOs were electroporated into OEPs at ss5-6, and otic tissue was analysed after 4.5 and 9 hours corresponding to ss8-9 and ss11-12, respectively. Experiments were performed in triplicate and genes with a fold change ≥+/-1.5 and a p-value ≤0.05 were considered to be deregulated (TableS2).

Several genes are rapidly upregulated after Lsd1 knockdown. These include the Notch targets *Hes4* and *Hey1* (a direct negatively-regulated Lsd1 target (Wang et al, 2007), the Notch ligand *Jag1* and *Cbx4*, a component of the Lsd1-CtBP and the PRC2 corepressor complexes (Shi et al, 2003; Tsai et al, 2010; Wang et al, 2007) (Fig.3A).This is followed by increase of a number of lineage markers: the epidermal marker *Gata2* (Sheng & Stern, 1999), the neural crest genes *Snail1*, *Msx1* and *Id4* (Kee & Bronner-Fraser, 2001; Nieto, 2002; Streit, 2002), and the lens-specific transcripts *Pax6*, *Zeb2, L-Maf and C-Maf* (Bailey et al, 2006; Bhattacharyya et al, 2004; Lecoin et al, 2004; Sheng et al, 2003) (Fig.3B). These data suggest that Lsd1 normally represses these transcripts and may prevent the switch of OEPs to other fates. By contrast, Lsd1 knockdown leads to loss of either OEP-specific genes (e.g. *Sox8*, *Pax2*, *Zbtb16*, *Etv4*) or genes that are already expressed at PPR stages

(e.g. *Foxi3*, *Gbx2*, *Spry1/2*, *Six1*, *Eya2*, *Znf385c*) with *Sox8*, *Pax2* and *Spry2* showing the greatest fold change (Fig.3A). Most of these genes are dependent on FGF signalling (Chen et al, 2017; Yang et al, 2013), and continue to be downregulated 9 hours after Lsd1 knock down, when additional downstream genes such as *Sox10* (a target of both *Sox8* and *Etv4*) (Betancur et al, 2011) are also reduced. These data indicate that Lsd1 is critically required to maintain the transcriptional profile of OEPs, while simultaneously preventing alternative fates.

To validate the NanoString data and as an alternative approach to perturb Lsd1 function, we cultured OEP explants in the presence of tranylcypromine (TCP) – a well-documented Lsd1 inhibitor (Schmidt and McCafferty, 2007) – and then performed qPCR for selected genes. This approach verifies the downregulation of OEP and posterior PPR genes at ss8-9 (Fig.3C, *Sox8*, *Pax2*, *Etv4*, *Zbtb16*, *Foxi3*, *Znf385c*) but not the upregulation of other genes (Fig.S1B). There is no increase in activated Caspase 3 in Lsd1-MO targeted OEPs (Fig.S4), indicating that reduction of Lsd1 does not promote apoptosis but that instead, OEPs lose their identity. Analysing OEP markers by *in situ* hybridisation 9-12 hours post MO electroporation reveals a complete loss of *Pax2* (n=8, Fig.2C-c), *Zbtb16* (n=6, Fig.2D-d) and *Etv4* (n=6, Fig.2E-e) after Lsd1 knockdown, while upregulated genes cannot be verified (Fig.S1C, C'). Interestingly, although Lsd1-MO only target the ectodermal layer, *Etv4* expression is also affected in the underlying mesoderm and endoderm, pointing to reciprocal signalling between these layers and the placode. The epibranchial expression of *Pax2* is also abolished (Fig.2c), and other epibranchial markers like *Foxi2* are reduced (n=5, Fig.S2D-d"). Morphologically, the placode is thinner, having lost the characteristic epithelial thickening compared to the control side (Fig.2c-e; inset in Fig2c; Fig.S3I-L). Only cells that escape being targeted by Lsd1-MO maintain *Pax2* expression and coalesce to form a hypoplastic cup or vesicle (n=8, Fig.S2A-a; Fig.S3E-H), whereas control morpholinos (Ctrl-MO) do not affect normal placode involution into a cup (n=6, Fig.S2B, B') or vesicle (n=4, Fig.S2C, C'). *Pax2* expression, loss of epithelial thickening as well as the late morphological

phenotype can be rescued by co-electroporating Lsd1-MO with full length human Lsd1-RFP (Fig.S3). Together, these data strongly support a role for Lsd1 in maintenance of the OEP transcriptional state and suggest that in this context it may act as a coactivator of OEP genes that determine OEP fate.

Lsd1 changes H3K9 modifications at OEP gene promoters

If Lsd1 functions as a direct coactivator of OEP and PPR genes, it should associate with their promoter regions to modify histone tails. To investigate this, we performed Lsd1 ChIP-qPCR on otic placodes and found that the promoters of *Sox8*, *Pax2*, *Etv4* and *Zbtb16* were bound by Lsd1, while promoters of *Spry2*, *Foxi3*, *Six1* and *Znf385c* did not show any significant Lsd1 recruitment (Fig.4A, Fig.S5). Thus, OEP genes are direct Lsd1 targets, while PPR factors are likely to be regulated indirectly.

As a coactivator, Lsd1 acts by demethylating the repressive H3K9me2 found predominantly at silent promoters (Barski et al, 2007). H3K9 has a dual function at gene promoters, with opposing roles depending on its modification: acetylation is associated with gene activation, whereas methylation rapidly silences gene expression (Karmodiya et al, 2012). Thus, H3K9me2 demethylation by Lsd1 (Metzger et al, 2016; Metzger et al, 2005) allows H3K9 acetylation, thereby maintaining an open chromatin state, while H3K9me2 accumulation in the absence of Lsd1 prevents H3K9ac and the chromatin remains closed (Fig.4B). Indeed, when Lsd1 is knocked down using MOs or inhibited by TCP, H3K9me2 increases in otic placode cells (Fig.S6). Importantly, using ChIP-qPCR for H3K9me2 and H3K9ac, we find that Lsd1 inhibition in OEP explant cultures by TCP results in an increase in H3K9me2 (Fig.4C) and a corresponding decrease in H3K9ac (Fig.4D) at the promoters of *Sox8*, *Pax2*, *Etv4* and *Zbtb16*. These data suggest that Lsd1 is normally recruited to the promoters of active OEP genes to prevent the accumulation of H3K9me2 and the loss of H3K9ac, thus allowing their continued expression.

cMyb interacts with Lsd1 and recruits it to target promoters

Lsd1 itself lacks the ability to bind DNA or to recognise the H3 tail within nucleosomes (Shi et al, 2005). How is Lsd1 recruited specifically to the promoters of OEP genes? As a corepressor, Lsd1 interacts with the cofactor of REST (CoREST) via its chromatin-interacting SANT2 domain (Ballas et al, 2005; Lee et al, 2005; Shi et al, 2005), or with SNAG-domain transcription factors (such as Snail1/2 and Gfi1) (Lin et al, 2010; Saleque et al, 2007), which recruit Lsd1 to their respective target genes. To investigate which factor is responsible for recruiting Lsd1 to the promoters of *Sox8*, *Pax2*, *Etv4* and *Zbtb16*, we performed a motif enrichment analysis on these promoters using RSAT (Fig.5A; TableS3). To narrow down potential Lsd1 partners in the ear, we removed factors belonging to the general transcriptional machinery and factors that are not expressed in OEPs. This strategy eliminates SNAG domain factors and hormone nuclear receptors, while returning Sox8, Pax2 as well as the SANT-domain containing coREST (via the presence of REST motif) and Myb as highly enriched motifs. Of those, Myb emerges as the top candidate to recruit Lsd1 to OEP gene promoters. Myb motifs are enriched in all four promoters (Fig.5A), *cMyb* is expressed specifically in OEPs at ss5-6, just prior to *Lsd1* (Betancur et al, 2011), it contains a SANT domain, and together with its transactivation domain, it can function as a coactivator or a corepressor (Boyer et al, 2004). Morpholino-mediated knockdown of cMyb in OEPs at ss3-4 leads to the loss of Lsd1-targets like *Pax2* (n=6, Fig.S7A-b) and *Zbtb16* (n=5, Fig.S7B, B'), but not of Lsd1 expression (Fig.S7C-J).

To assess whether cMyb and Lsd1 physically interact, we used lysates from neuroblastoma cells which express these proteins in abundance (Fig.5B) to perform co-immunoprecipitation. While cMyb antibodies do not work in immunoprecipitation, Lsd1 antibodies successfully precipitate Lsd1 protein (Fig. 5B''). We therefore used Lsd1 antibody for immunoprecipitation followed by western blotting with cMyb antibody; this reveals that both proteins interact (Fig.5B'). We then asked whether cMyb is required for Lsd1 binding to

the *Sox8*, *Pax2*, *Etv4* and *Zbtb16* promoters. Control or cMyb-MOs (Betancur et al, 2011) were electroporated into OEPs at ss3, targeted cells were harvested 10.5 hours later at ss10 and processed for Lsd1 ChIP followed by qPCR for *Sox8*, *Pax2*, *Etv4* and *Zbtb16* promoter regions. While Lsd1 binds all four promoters in controls, this is strongly reduced when cMyb is knocked down (Fig.5C). Together, our data suggest that cMyb and Lsd1 proteins physically interact and that cMyb recruits Lsd1 to the promoters of the OEP genes *Sox8*, *Pax2*, *Etv4* and *Zbtb16*. In turn, this keeps H3K9 demethylated and allows its acetylation thus maintaining OEP gene expression.

Discussion

While the epigenetic mechanisms that maintain stem cell pluripotency and the exit from this state *in vitro* are fairly well characterised, their role in controlling cell fate decisions and maintaining cell identity in normal embryonic development is much less understood. Here we take advantage of the recently established gene regulatory network for ear development to investigate the epigenetic mechanisms that maintain ear precursors in a progenitor state prior to lineage commitment. Ear commitment is a gradual process that begins with OEP specification from a pool of sensory progenitors. Exposed to a changing extrinsic environment as morphogenesis proceeds, OEPs must stabilise their identity and retain competence to respond to signals that ultimately commit them to their fate (Fig.6A). Here, we establish a central role for the demethylase Lsd1 in this process, driven by the transcription factor cMyb (Fig.6B,C).

Lsd1 maintains the OEP module at the top of the inner ear regulatory network

The control of OEP specification and their subsequent commitment to either the otic or epibranchial lineages involves a hierarchical organisation of gene regulatory networks and sub-networks. These network modules are interconnected and embedded within them are the signalling inputs that direct cell behaviour (Chen et al, 2017). Because of the hierarchical network organisation and the crosstalk between regulatory modules, failure to maintain components of just one module will essentially shutdown the entire developmental programme. This is precisely what we observe in the absence of Lsd1 function. *Lsd1* expression is activated in OEPs after specification, and its expression persists in otic cells as they commit to their fate. Therefore, by directly maintaining the expression of key players in the OEP module (*Sox8*, *Pax2*, *Etv4* and *Zbtb16)* that precede its own expression, Lsd1 becomes part of a sub-network that acts like a central hub for the entire ear cascade. Interestingly, these Lsd1 targets are dependent on FGF signalling for their initial expression,

and Lsd1 is known to regulate both Wnt and FGF signalling pathways (Chen et al, 2016; He et al, 2016; Huang et al, 2017; Huang et al, 2013; Lei et al, 2015; Takeuchi et al, 2015).

In the zebrafish lateral line, Lsd1 is required to activate *Etv4*, *Axin2* and *Tcf7l2* to promote supporting cell proliferation upon hair cell damage (He et al, 2016), whereas it downregulates *Etv2* to promote commitment of hematopoietic progenitor cells to their fate (Takeuchi et al, 2015). Furthermore, in cancer cells, Lsd1 promotes the Wnt pathway by repressing negative regulators of Wnt/β-Catenin signalling like *APC*, *Prickle*, *Sfrp5* and *Dkk1* (Huang et al, 2017; Huang et al, 2013; Lei et al, 2015). In contrast, Lsd1 can also negatively regulate Wnt signalling by repressing Wnt pathway components such as *Fzd1/2* to promote mesenchymal stem cell differentiation into brown adipocytes (Chen et al, 2016). In the ear, Wnt signalling is required for the transition of OEPs to committed otic cells (Freter et al, 2008; Jayasena et al, 2008; Park & Saint-Jeannet, 2008). Thus, it is possible that maintenance of the OEP module by Lsd1 is necessary for OEPs to retain competence to respond to Wnt signalling and that Lsd1 directly or indirectly promotes the expression of Wnt pathway components.

Virtually nothing is known about the upstream regulators of *Lsd1*, although in our NanoString data, the expression of *Lsd1* itself is lost upon Lsd1-MO knockdown. As the expression of *Sox8*, *Pax2*, *Etv4* and *Zbtb16* in OEP cells precede *Lsd1* expression, it is likely that one or more of these factors regulate *Lsd1* expression.

The Lsd1-cMyb coactivator complex prevents deactivation of OEP promoters

Lsd1 is a demethylase found in both activator and repressor complexes. While the latter is well studied (Lin et al, 2010; Shi et al, 2005), its role as a coactivator is poorly defined. When Lsd1 is recruited by hormone nuclear receptors to their target promoters, its substrate specificity switches from H3K4me2 to H3K9me2 (Metzger et al, 2016; Metzger et al, 2005). In turn, demethylated H3K9 provides a substrate for histone acetyltransferases, with H3K9ac at promoter regions being a hallmark for actively transcribed genes. In addition, the

neuronal-specific isoform Lsd1+8a targets H3K9 for demethylation in the absence of nuclear receptors (Laurent et al, 2015). However, this isoform is only found in mammals, but is absent in other vertebrates like chick, fish and frog (Laurent et al, 2015; Zibetti et al, 2010). Our experiments reveal that Lsd1 can indeed switch substrate preference to act as a coactivator in the presence of cMyb.

In the otic placode, cMyb cooperates with Sox8 and Etv4 to control the expression of the late otic marker *Sox10*: cMyb knockdown results in *Sox10* downregulation and the loss of placode thickening (Betancur et al, 2011). This phenotype is remarkably similar to Lsd1 knockdown. Here, we show that Lsd1 and cMyb physically interact and that Lsd1 recruitment to its target promoters depends on the presence of cMyb. This finding suggests that both are part of the same molecular machinery that controls otic lineage commitment. As *Pax2* is expressed prior to *Lsd1*, we propose that Lsd1 can only function in OEP maintenance when both cMyb and Lsd1 are present. *Pax2* is initiated by FGF and one study suggests that this pathway must be downregulated (Freter et al., 2012) for cells to commit to the otic lineage. This coincides with the onset of Lsd1 expression, and it is therefore possible that Lsd1 acts to maintain *Pax2* when FGF signalling begins to decline.

At promoter regions of OEP genes, Lsd1 prevents accumulation of the repressive mark H3K9me1/2 through its demethylase activity allowing H3K9ac accumulation. cMyb is likely to play a key role to facilitate this. Unlike the chromatin-interacting SANT domain, which facilitates histone deacetylation by HDACs (Boyer et al, 2004), the cMyb SANT domain binds DNA and cMyb recruits the histone acetyltransferase p300 via its transactivation domain (Mo et al, 2005). In contrast, high levels of H3K9 methylation are essential along the gene body of actively transcribed genes and this is facilitated by histone methyltransferases like Setdb1 or Ehmt2 (also known as G9a) (Layman & Zuo, 2014; Ooi & Wood, 2007). During adipogenesis Lsd1 opposes the activity of Setdb1 at target promoters to prevent premature differentiation (Musri et al., 2010). However, the precise mechanism of how this occurs and whether Lsd1 modifies Setdb1 itself or whether there is negative feedback

between the complexes remains elusive. We therefore suggest that the Lsd1-cMyb-p300 coactivator complex at the promoter region of OEP genes is crucial to counteract HDAC and EHMT activities, and as a result prevents deactivation of OEP transcripts.

In addition, Lsd1 may also be part of a repressor complex as HDACs and REST/coREST are present in OEPs, albeit being ubiquitously expressed in the embryo (Chen et al, 2017). In this context Lsd1 may be important to repress non-otic genes, which may in turn prevent ear development. We have previously shown that in the absence of additional signals, sensory progenitors in the PPR activate the lens programme through expression of the early marker *Pax6* and late lens genes like *L-maf* and *δ-crystallin*. Lsd1 knockdown leads to the upregulation of lens genes, although at very low levels, and it is therefore tempting to speculate that Lsd1 is part of the machinery that prevents the lens default fate (Bailey et al, 2006). However, the upregulation of these genes is not maintained, possibly due to inhibitory signals, and therefore the cells do not progress towards lens development. Together our findings suggest that the Lsd1-cMyb-p300 coactivator complex lies at the heart of a mechanism that maintains OEP identity, and that Lsd1 is a molecular cell fate switch that ensures the execution of the ear programme.

Epigenetic regulation of cell maintenance and fate commitment in the embryo

Much of our understanding of the epigenetic regulation of cell fate decisions centres around maintenance of embryonic, neural or hematopoietic stem cells (Adamo et al, 2011; Foster et al, 2010; Sun et al, 2010; Takeuchi et al, 2015; Thambyrajah et al, 2016), cancer cells (Huang et al, 2017; Lei et al, 2015) and progenitor cell differentiation in the fetal or postnatal retina (Popova et al, 2016), anterior pituitary (Wang et al, 2007) or nervous system (Ballas et al, 2005). In all these cases, Lsd1 acts as a corepressor to either maintain stem cell identity or allow differentiation towards a particular fate, mostly targeting H3K4me1 at enhancer or H3K4me2 at promoter regions. However, there is limited evidence of the crosstalk between genetic and epigenetic regulation of cell fate in the developing embryo. To our knowledge,

this is the first report that demonstrates a coactivator role for Lsd1 in cell fate maintenance. Our findings place Lsd1 within a gene regulatory network and show how a single epigenetic modifier maintains a network module that is in turn crucial to promote the ear programme. Many factors within this module are involved in other biological processes, cancer and/or mutated in human syndromes. Thus, from a therapeutic perspective, this study may provide insights into the mechanisms underlying the human syndromes caused by mutations in *LSD1* (Chong et al, 2016; Tunovic et al, 2014) such as cleft palate, psychomotor retardation and distinct facial features (CPRF; OMIM # 616728) as well as overlapping features with Kabuki syndrome (OMIM # 300867).

Materials and Methods

Chromatin immunoprecipitation (ChIP)

Otic tissue collected in nuclear extraction buffer (10mM Tris-HCL pH7.5, 3mM $CaCl_2$, 250mM Sucrose, 0.5% NP-40, 0.25% TritonX-100, supplemented with 1mM DTT and protease inhibitors, PI) was homogenised using a dounce homogeniser on ice, cross-linked in 0.9% formaldehyde, quenched with 125mM glycine. Nuclei were pelleted, washed in PBS (with DTT/PI) and lysed in SDS lysis buffer (50mM Tris-HCl pH8, 1% SDS, 10mM EDTA pH8, PI) on ice for 1hr to release cross-linked chromatin. The chromatin was then sonicated using SorvicsVibra Cell and probe CV18 on ice in ChIP buffer without DTT (16.7mM Tris-HCl pH8, 0.% SDS, 1.2mM EDTA pH8, 167mM NaCl) to obtain DNA fragments of 0.2-1kb. Fragmented chromatin was diluted in ChIP buffer with 1mM DTT and PI. Protein A magnetic beads (Invitrogen 10002D) were blocked in 0.5% BSA/PBS, incubated with 5µg of antibody at 4°C O/N on a rotator, collected using a magnetic stand (Invitrogen 12321D). Immunoprecipitation was performed by resuspending antibody-beads in fragmented chromatin at 4°C O/N on a rotator, then repeatedly washed in RIPA buffer (50mM HEPES-KOH pH8, 500mM LiCl, 1mM EDTA pH8, 1% NP-40, 0.7% sodium deoxycholate, PI) followed by TE/50mM NaCl wash. Elution was performed at 65°C for 15min with elution buffer (50mM Tris-HCl pH8, 10mM EDTA pH8, 1% SDS) and reverse cross-linked at 65°C O/N together with input, then treated with RNAse A for 1hr at 37°C followed by 0.2µg/ml proteinase K at 55°C for 1hr. DNA was recovered by phenol-chloroform extraction and ethanol precipitation. RT-qPCR was performed using SYBR green and AriaMx Real-Time PCR System (Agilent Technologies). Antibodies were from Abcam: Lsd1 (ab17721), H3K9ac (ab4441), H3K9me2 (ab1220).

Co-immunoprecipitation (Co-IP)

For immunoprecipitation assays, nuclear cell extracts from neuroblastoma cells were

precleared in 0.5% BSA for 1hr on ice then with protein A magnetic beads for 30min at 4°C to reduce non-specific binding and background. Precleared lysates were separated from the beads and incubated with 5µg of antibody (anti-Lsd1, Abcam ab17721) in Co-IP buffer (20mM Tris-HCl pH7.5, 100mM NaCl, 0.2% TritonX-100, 0.2% NP-40, 2mM β-mercaptoethanol, 0.2% sodium deoxycholate, 1mM DTT, PI) at 4°C O/N, then with beads for 4hrs at 4°C followed by repeated washes in wash buffer (20mM HEPES pH7.5, 1.25mM $MgCl_2$, 0.1mM EDTA pH8, 10% glycerol, 0.2% NP-40, 100mM KCl, 1mM DTT, PI). Beads were collected, resuspended in 2x SDS loading buffer, boiled for 5min at 95°C and run on SDS-PAGE gel for WB.

Western blot (WB)

Protein lysates from neuroblastoma cells were separated by SDS-PAGE and transferred onto PVDF membranes using a Bio-Rad Trans-blot Turbo transfer system. After blocking, the membranes were incubated in primary antibody (anti-cMyb, Abcam ab45150 or anti-Lsd1) overnight at 4°C followed by PBST washes and incubation with HRP-conjugated secondary antibody for 2 hours. Signals were visualised using Bio-Rad Clarity Western ECL Substrate and Bio-Rad ChemiDoc Touch imaging system.

ChIP-qPCR data and statistical analysis

Input Ct values were adjusted for the dilution factor and ΔCt calculated by normalising Ct values to the adjusted input: ΔCt=Ct(input)-Ct(IP). The % input was calculated as % Input = $100x2e^{-\Delta Ct}$. Fold enrichment was calculated using % input of IP/IgG: (ΔCt(IP)/ΔCt(IgG). Error bars represent the standard error. Unless otherwise indicated, differences between experimental groups were compared using an unpaired two-tailed Student's t-test and a P-value of ≤0.05 was considered statistically significant.

Fluorescence quantification

ImageJ was used to measure the integrated density (fluorescence intensity) in the region of interest for both electroporated and drug treated samples. For electroporated cells, the mean fluorescence intensity of targeted cells/Hoechst was compared to non-targeted cells/Hoechst, whereas for drug-treated vs untreated samples, the entire placode/Hoechst was measured. To correct for different exposure levels between channels and individual samples, the integrated density from the mean of 3 background areas was also calculated. The formula used to estimate the corrected total cell fluorescence (CTCF) was as follows: Integrated density – (Area of selection x Mean fluorescence of background reading). Statistical significance was estimated using unpaired t-test.

Motif enrichment analysis

Transcription factor binding motifs were predicted using JASPAR database (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) and P-Match program from Transfac database (http://www.gene-regulation.com/pub/programs.html). A profile matrix was generated to run a pattern matching scan using RSAT (http://rsat.sb-roscoff.fr/) on the promoters for enrichment against the background (chicken genome).

Pharmacological inhibition

Dissected OEP tissues were cultured in collagen with trans-2-phenylcyclopropylamine (tranylcyclopromine, TCP, at 25µm working concentration prepared in sterile water) in culture medium. Explants were incubated with drug (or no drug control) for 30 minutes at room temperature before culturing for either 4.5 or 9 hours. For sectioning and immunofluorescence, the whole embryo or head was cultured in the presence or absence of drug for 12 hours on polycarbonate membranes (Corning, 3422).

***In ovo* electroporation and tissue dissection**

Fertilised hen's egg (Winter Farm, Hertfordshire) were incubated in a humidified incubator at

38°C to reach ss5-6. Eggs were windowed and embryos visualised with India ink (Pelikan; diluted 1:5 in Tyrodes saline). The positive (platinum) electrode was placed under the embryo at hindbrain (r4) level, DNA/morpholino injected below the vitelline membrane, negative (tungsten) electrode placed on top and current applied using an Intracel electroporator (settings: 5 volts, 5 pulses, 50ms, 10ms duration). Tyrodes saline supplemented with penicillin streptomycin was applied to the embryo, egg resealed with tape and incubated to the appropriate stage. Placodes were dissected as described previously (Anwar et al, 2017; Chen et al, 2017).

***In situ* hybridisation and immunofluorescence**

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and *in situ* hybridisation performed using DIG-labelled riboprobes. For antibody staining, embryos were embedded in paraffin wax or gelatine and sectioned to 8µm. Primary antibodies were: anti-H3K9ac (Abcam ab4441) 1:100; anti-H3K9me2 (Abcam ab1220) 1:100; anti-FITC 488 (Thermo Scientific 4-4-20) 1:500; anti-mCherry (Abcam ab167453) 1:500; anti-cleaved caspase 3 (Cell Signalling 9964B) 1:300 and anti-Lsd1 (Abcam ab17721) 1:300. Secondary antibodies were: goat anti-rabbit or anti-mouse Alexa Fluor 568 (1:1000, Invitrogen) and Alexa Fluor 635 (1:500, Invitrogen).

Morpholinos and DNA constructs

Fluoresceinated morpholino antisense oligonucleotides were obtained from Gene Tools (USA) with standard Ctrl-MO (5’ – CCTCTTACCTCAGTTACAATTTATA – 3’) from manufacturer and cMyb-MO (5’ – ATGGCCGCGAGCTCCGCGTGCAGAT – 3’) published in Betancur et al., 2011. Both Lsd1 morpholinos were splice morpholinos. Lsd1-MO 1: (5’ – TTTGTGAAACTCACCAAGTCCTGTT – 3’) deletes exon 8 (enzymatic domain). Lsd1-MO 2 (5’ – ATCACTGGACAAAATCTGACCTTGT – 3’) deletes exon 11 (substrate binding domain). All morpholinos were used at 1mM final concentration. Lsd1-RFP expression constructs were generated by subcloning the human Lsd1 (gift from Prof. Yang Shi) open reading frame into pCAB.IRES.RFP vector.

Quantitative RT-PCR and NanoString nCounter analysis

Preparation of tissue for qPCR and NanoString analysis were conducted as previously described (Anwar et al, 2017; Chen et al, 2017).

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Author contributions

M.A. and A.S. designed the project; M.A. performed the experiments and data analysis; M.A. and A.S. wrote the manuscript.

Conflicting interests

The authors declare no conflict of interest.

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Figure Legends

Fig.1. *Lsd1* is expressed in progenitor cells after their specification. At ss5, *Lsd1* is expressed in the neural tube (nt) and mesoderm (m) but not in otic-epibranchial progenitors (A, a; arrowhead). At ss8 (B, b) and ss13 (C, c), *Lsd1* is expressed in the otic placode and lateral ectoderm (arrowhead). The expression of *Lsd1* in OEPs begins at ss8-9 after OEP specification indicated by *Pax2* expression (D). *Sdha* was used as a housekeeping gene to determine the qPCR mRNA expression levels.

Fig.2. Lsd1 is necessary for otic placode and vesicle formation. (A-b') left and right side of the same embryo. (A) normal otic vesicle. (a) higher magnification of the area boxed in A. (a') cross section of region indicated by line in A. (B) Lsd1-MO electroporated cells do not form an otic vesicle – only non-targeted cells (inset; yellow arrow) do. (b) higher magnification of the area boxed in B. (b') cross section of region indicated by line in B. Asterisk: normal otic vesicle on the left side that can also be seen from the right side. The expression of OEP markers (C'-E'; c-e) is abolished on the side electroporated with Lsd1-MO (C-E; green). Black line: sectioned regions shown in c-e. Dashed boxes: in C-E' = OEP region on the electroporated side; in c = regions shown in insets at higher magnification.

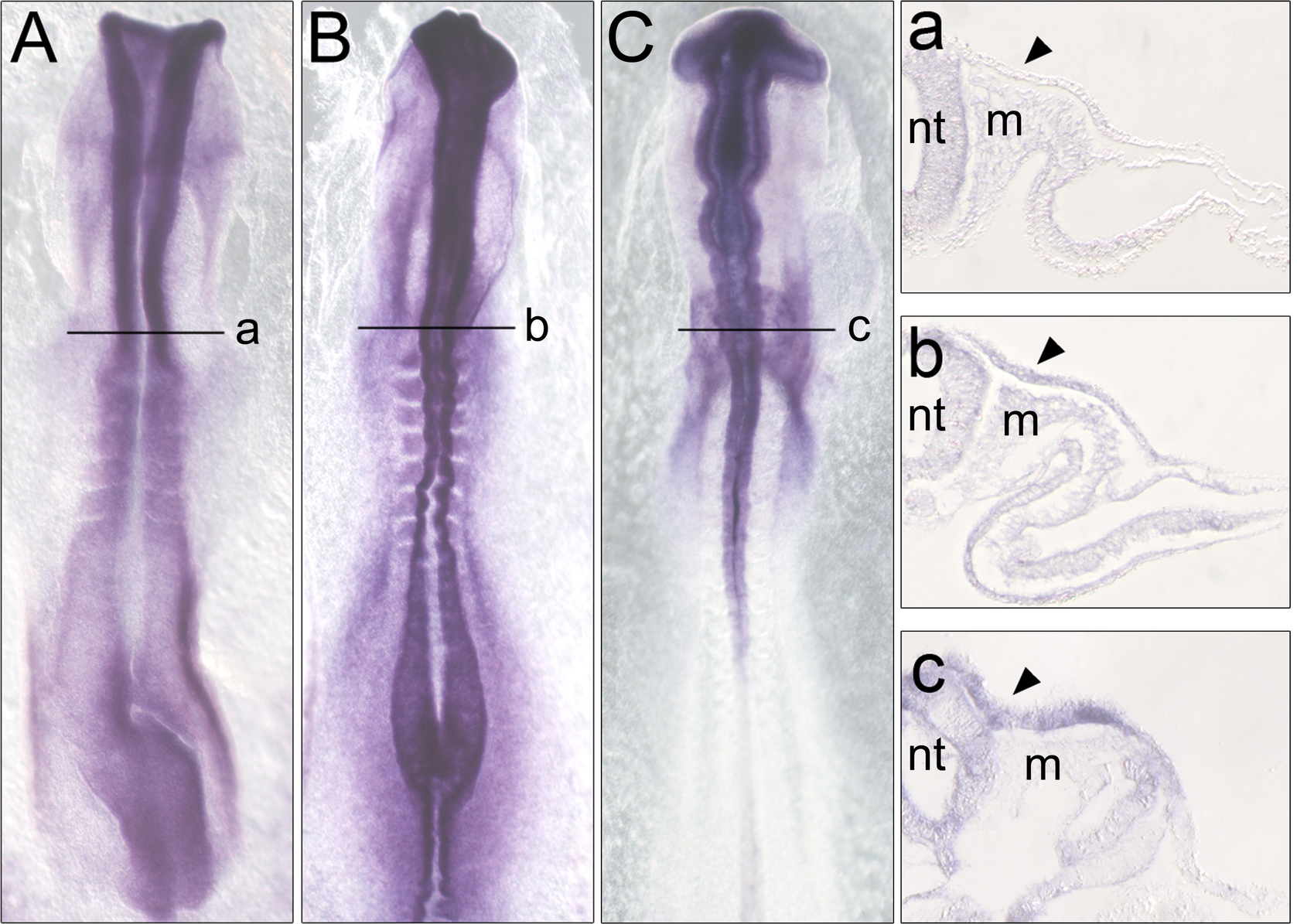
Fig.3. Differentially regulated genes following Lsd1-MO knockdown. (A, B) Volcano plot representing changes in gene expression by NanoString analysis following Lsd1 knockdown at late OEP (ss8-9) and lineage-committed placodal (ss11-12) stages. Genes affected at ss8-9 are potential immediate targets of Lsd1. (C) Validation of selected genes by qPCR following pharmacological inhibition of Lsd1 with TCP. Experiments were conducted in triplicates and *Sdha* was used for normalisation.

Fig.4. Lsd1 occupies the promoters of target genes to remove H3K9me2. (A) Lsd1 ChIP-qPCR on promoters of genes that are downregulated after Lsd1 inhibition. Bar diagram shows relative enrichment of Lsd1 binding compared to IgG controls. Lsd1 binds to the promoters of OEP, but not PPR genes. (B) To maintain active transcription, Lsd1 prevents accumulation of methyl groups on H3K9 thus allowing it to remain acetylated. In the absence of Lsd1, H3K9 methylation accumulates as H3K9ac is lost and the promoter is transcriptionally silenced. (C, D) ChIP for histone marks shows an increase of H3K9me2 at the target promoters following Lsd1 inhibition (C) and simultaneous loss of H3K9ac (D).

Fig.5. Lsd1 interacts with cMyb which recruits it to target promoters. (A) Motif enrichment analysis shows that Myb binding site is present in the promoters of all four OEP genes; cMyb is expressed at the appropriate time in the OEPs. (B) Neuroblastoma cell lines express Lsd1 and cMyb. (B', B'') Immunoprecipitation with Lsd1 antibody followed by cMyb western blot reveals Lsd1-cMyb interaction. (C) Lsd1 promoter occupancy is lost following MO-mediated knock-down of cMyb.

Fig.6. Model for an Lsd1-cMyb coactivator complex in progenitor cell maintenance. (A) OEPs arise from sensory progenitors (PPR) identified by a set of genes. FGF activates OEP genes in the PPR; *cMyb* is not induced by FGF but becomes expressed at the same time. In a transitory state, OEPs upregulate *Lsd1* and maintain their identity until they respond to new signals and commit to a particular lineage by activating additional fate-specific genes. (B) cMyb recruits Lsd1 to active OEP promoters already decorated with H3K4 methylation and H3K9ac. To maintain their expression, Lsd1 prevents H3K9 methylation at the promoter, thereby allowing H3K9 to remain acetylated, and restricts H3K9me2 to the gene body. (C) Inhibition of Lsd1 (1) results in the loss of H3K9ac and gain of H3K9me2 at the active promoters, which are rapidly silenced and OEP gene expression is lost. Loss of cMyb (2) prevents the recruitment of Lsd1 to the promoters leading to the same outcome as Lsd1

709 inhibition.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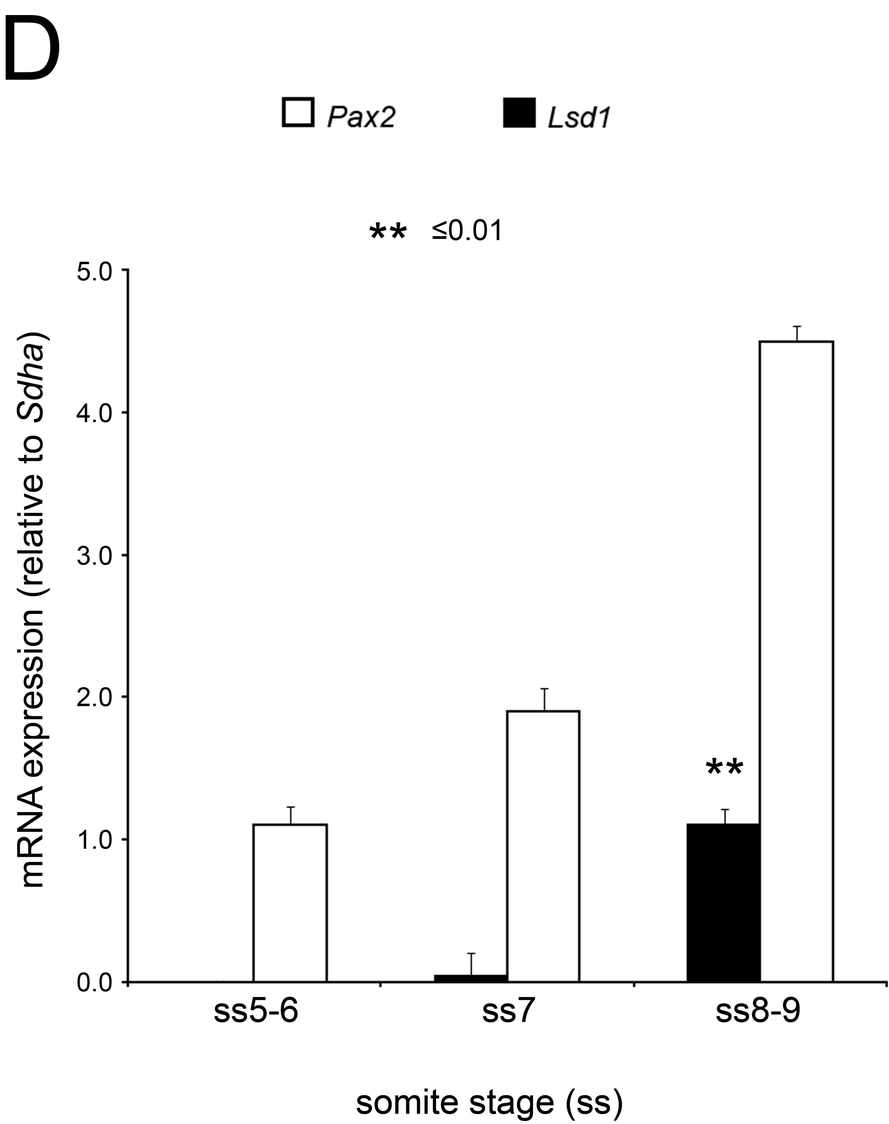
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a'

a

Left

a

a'

B

b'

b

Right

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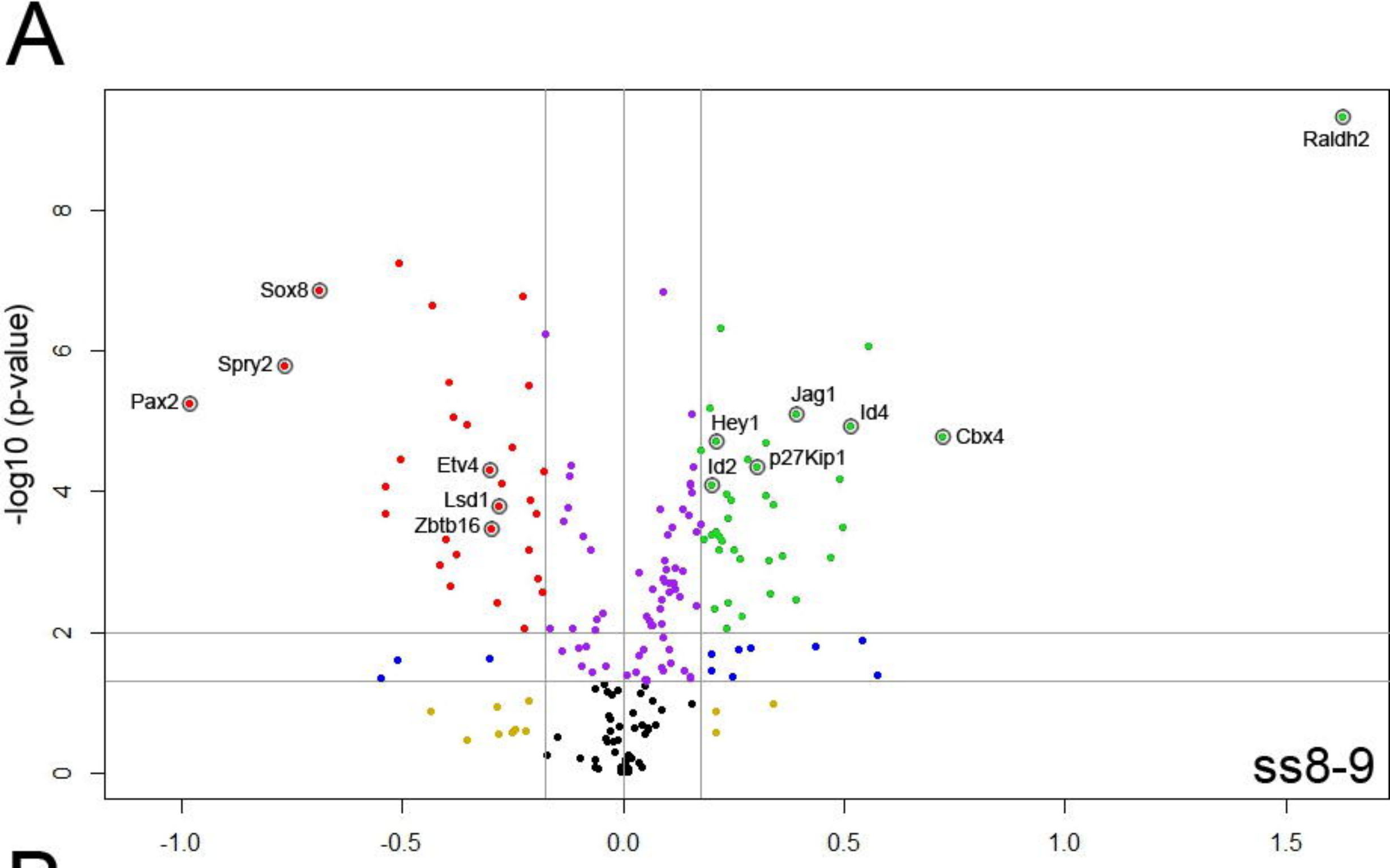
Raldh2
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Id4
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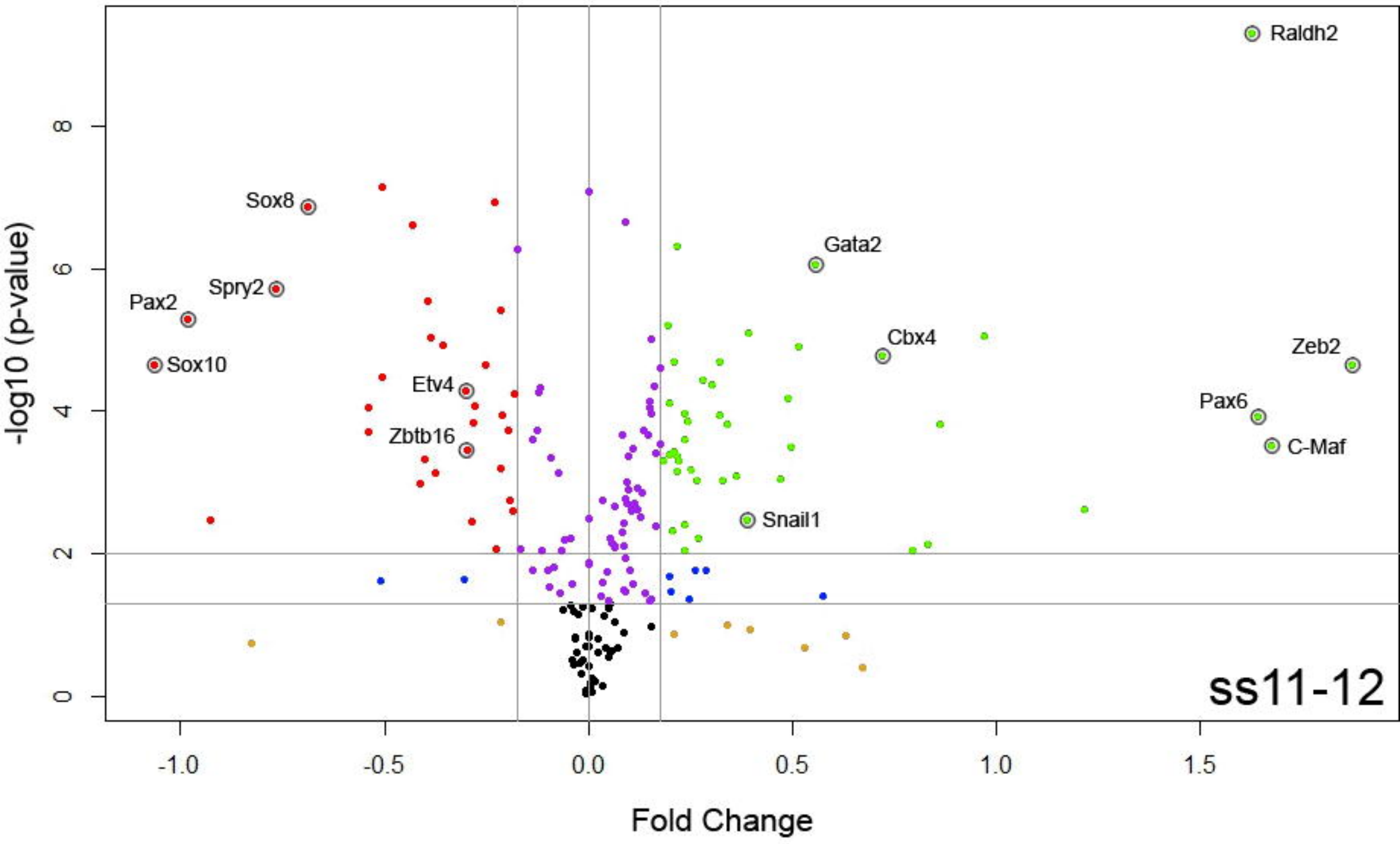
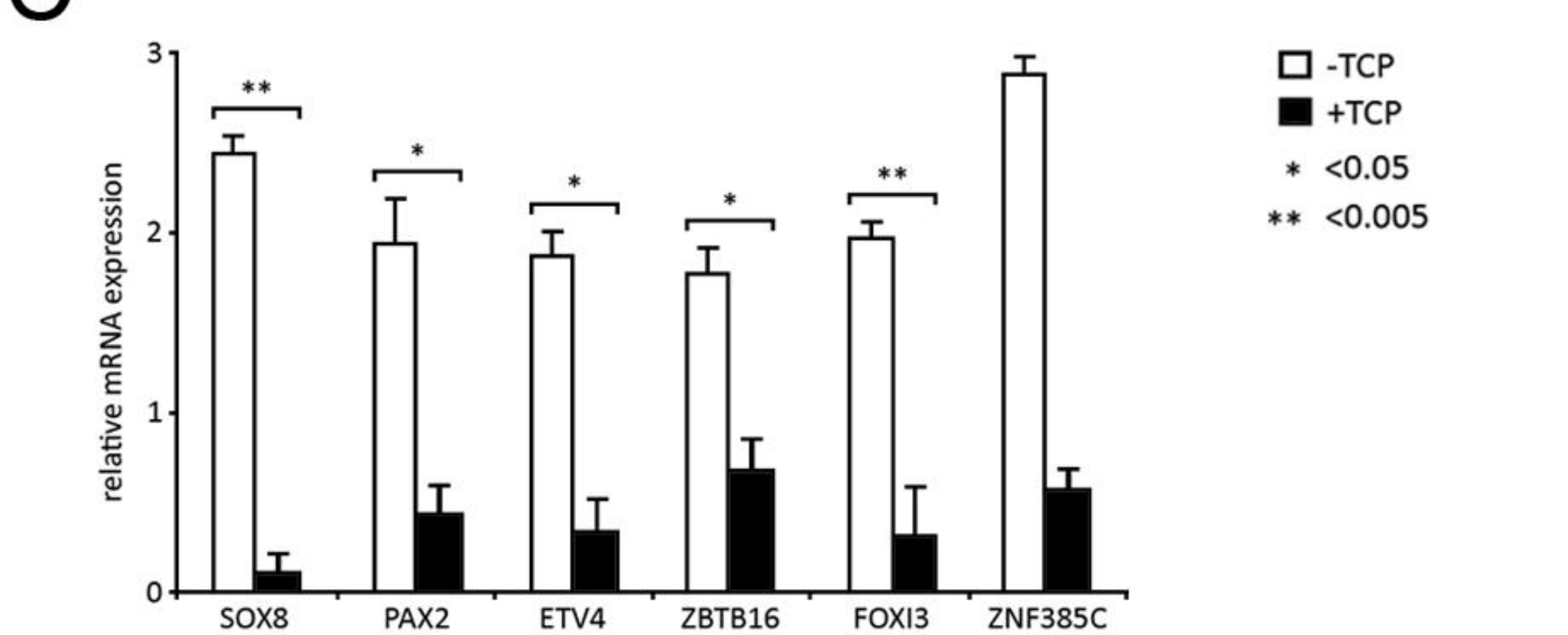
Cbx4
Etv4
Id2
p27Kip1
Lsd1
Zbtb16
-log10 (p-value)
0
2
4
6
8
ss8-9
-1.0
-0.5
0.0
0.5
1.0
1.5
Fold Change

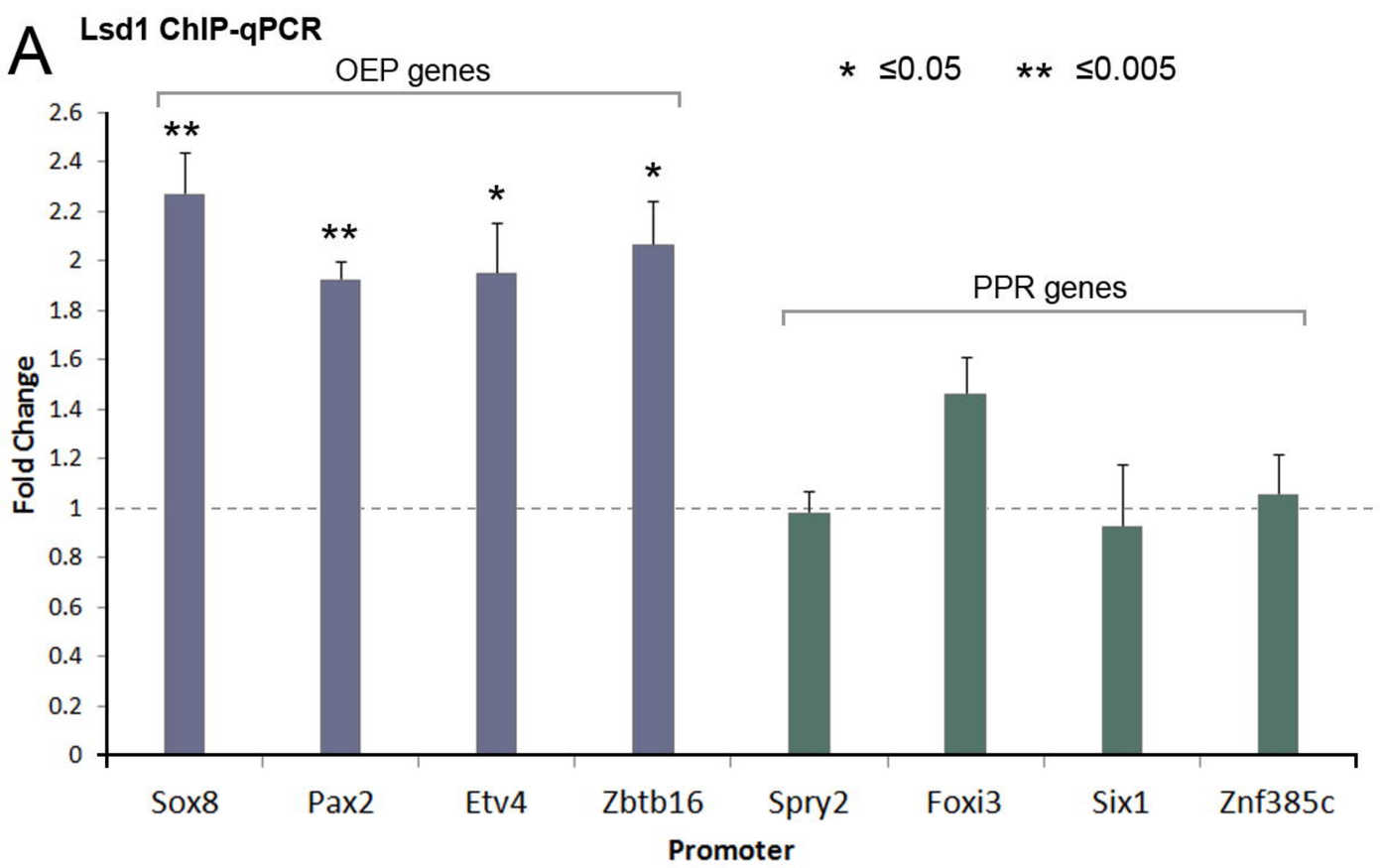
B


Raldh2
Sox8
Gata2
Spry2
Pax2
Cbx4
Zeb2
Sox10
Etv4
Pax6
Zbtb16
C-Maf
Snail1
-log10 (p-value)
0
2
4
6
8
ss11-12
-1.0
-0.5
0.0
0.5
1.0
1.5
Fold Change

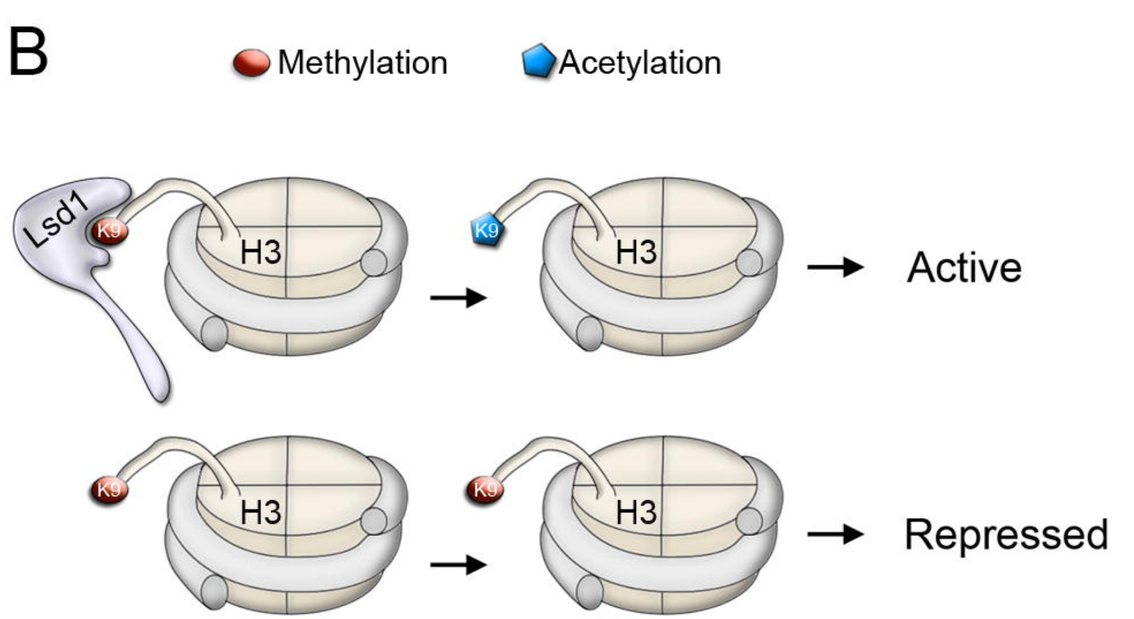
C


relative mRNA expression
0
1
2
3
**
*
*
*
**
SOX8
PAX2
ETV4
ZBTB16
FOXI3
ZNF385C
Genes
-TCP
+TCP
* <0.05
** <0.005

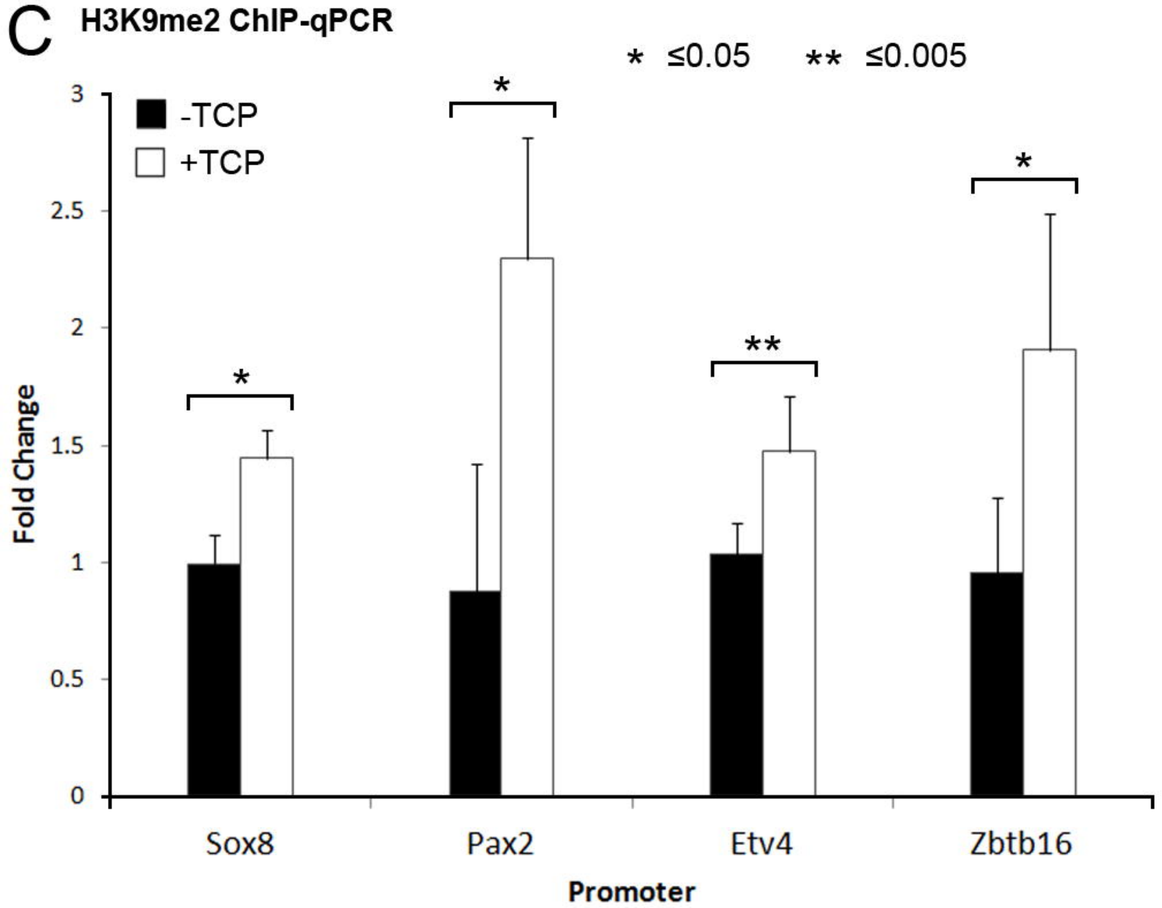
A


Lsd1 ChIP-qPCR
OEP genes
* ≤0.05 ** ≤0.005
PPR genes
Fold Change
Sox8
Pax2
Etv4
Zbtb16
Spry2
Foxi3
Six1
Znf385c
Promoter

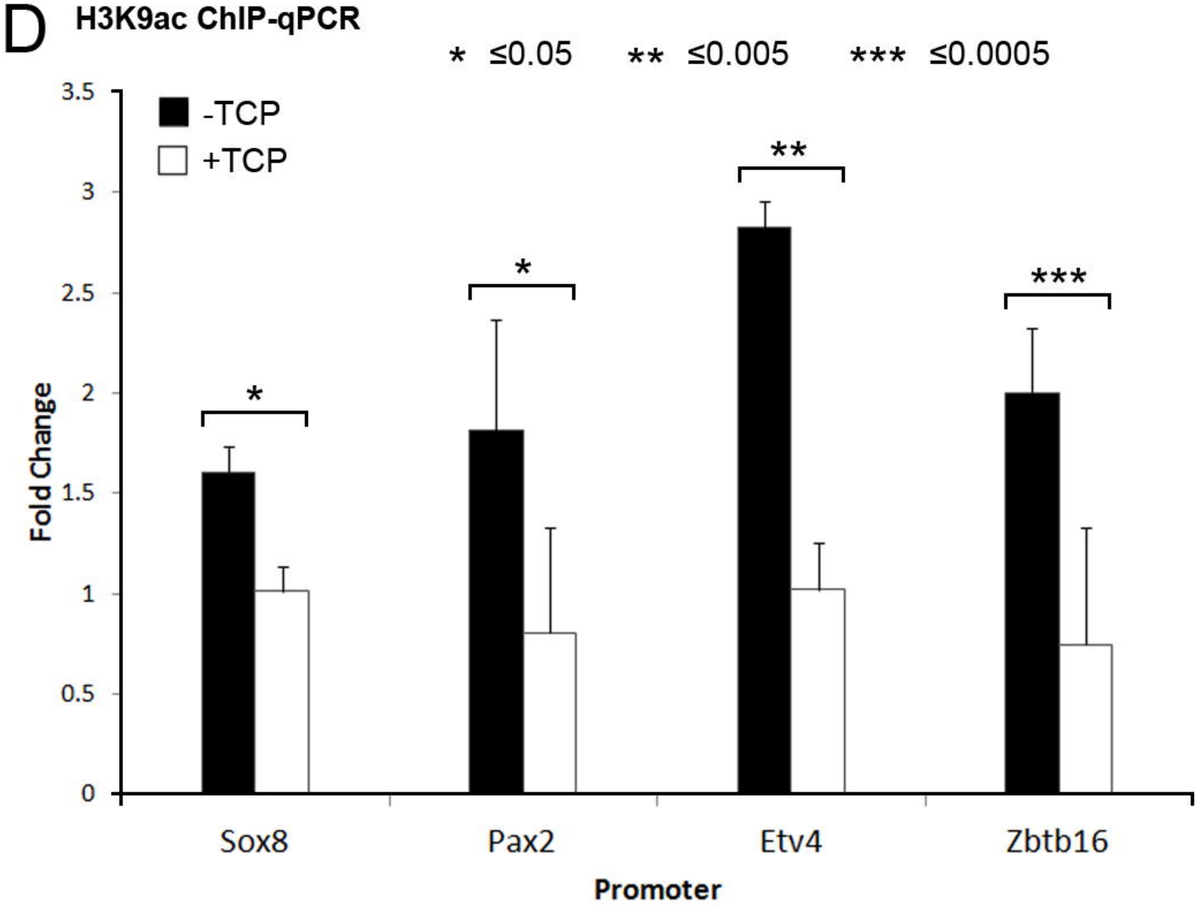
B


Methylation
Acetylation
Lsd1
K9
H3
Active
Repressed

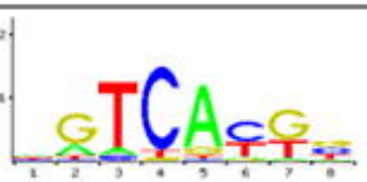
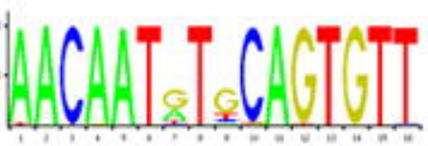
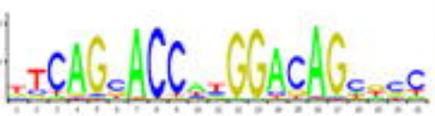
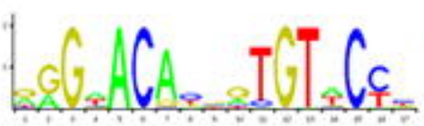
C

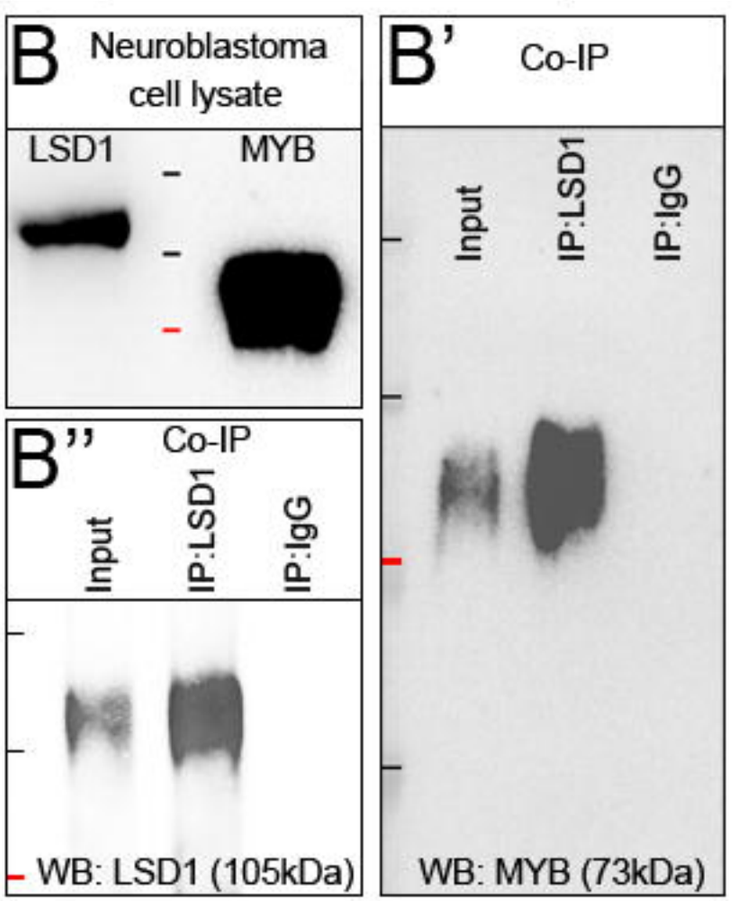

H3K9me2 ChIP-qPCR
* ≤0.05 ** ≤0.005
-TCP
+TCP
Fold Change
Sox8
Pax2
Etv4
Zbtb16
Promoter

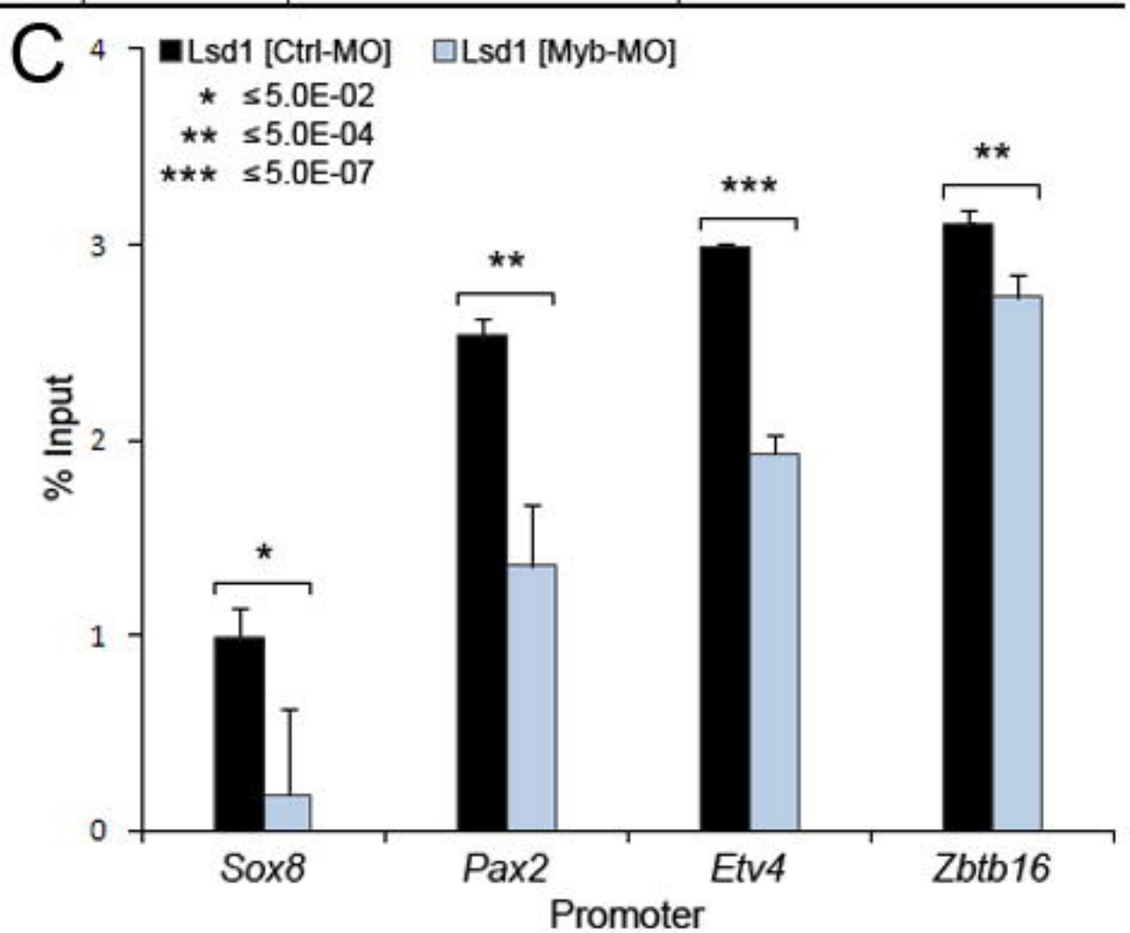
D


H3K9ac ChIP-qPCR
* ≤0.05 ** ≤0.005 *** ≤0.0005
-TCP
+TCP
Fold Change
Sox8
Pax2
Etv4
Zbtb16
Promoter

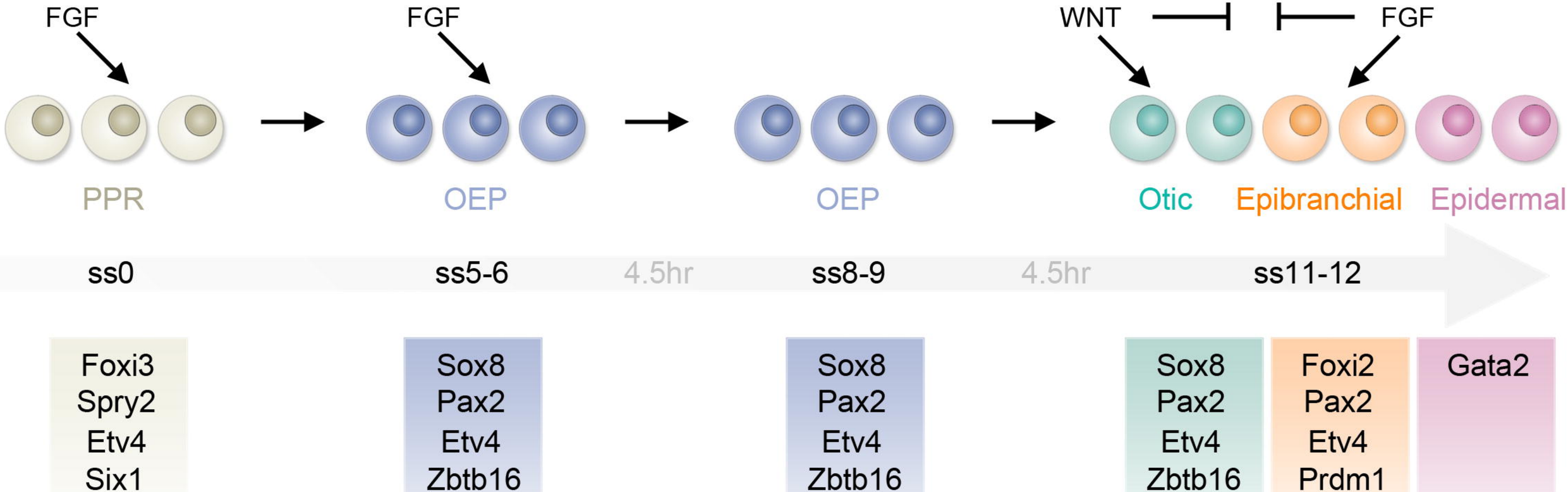
A

TF	TF Motif	Promoters	p-Value	Expressed in OEPs?	Protein Complex with Lsd1?	Reference
MYB		*Sox8* *Pax2* *Etv4* *Zbtb16*	4.10E-05 1.80E-05 2.80E-05 3.20E-05	Yes - 5-6ss	Unknown	Betancur et al., 2011
PAX2		*Pax2* *Zbtb16*	4.60E-05 7.60E-06	Yes - 5-6ss	Unknown	Groves and Bronner-Fraser, 2000
SOX8		*Sox8* *Pax2* *Etv4*	5.40E-05 9.10E-06 7.50E-05	Yes - 5-6ss	Unknown	McKeown et al., 2005
REST		*Pax2* *Zbtb16*	1.10E-05 1.40E-05	ubiquitous	Yes, interaction with CoREST	Lee et al., 2005; Shi et al., 2005
AR		*Sox8* *Pax2* *Etv4* *Zbtb16*	2.00E-05 9.10E-05 3.70E-05 2.90E-05	No	Yes	Metzger et al., 2005

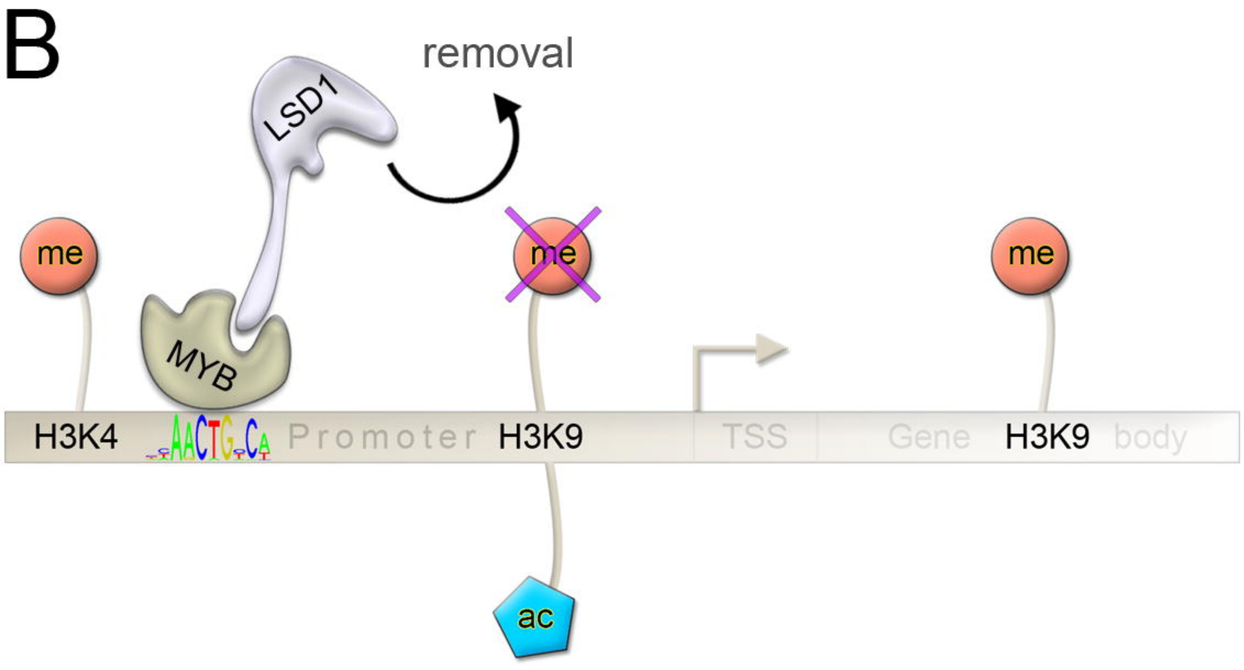




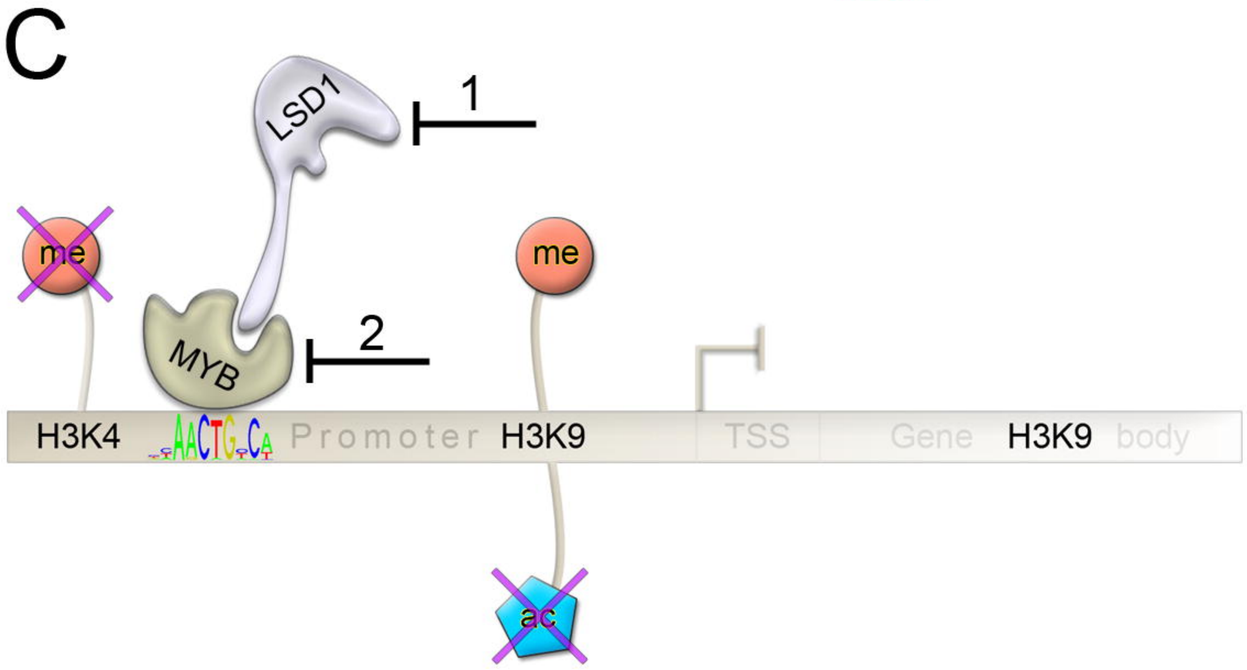
A


FGF
FGF
WNT
FGF
PPR
OEP
OEP
Otic
Epibranchial
Epidermal
ss0
ss5-6
4.5hr
ss8-9
4.5hr
ss11-12
Foxi3
Spry2
Etv4
Six1
Sox8
Pax2
Etv4
Zbtb16
cMyb
Sox8
Pax2
Etv4
Zbtb16
cMyb
Lsd1
Sox8
Pax2
Etv4
Zbtb16
cMyb
Lsd1
Foxi2
Pax2
Etv4
Prdm1
Vgll2
cMyb
Lsd1
Gata2

B


removal
LSD1
MYB
me
me
me
H3K4
Promoter
H3K9
TSS
Gene
H3K9
body
ac

C


LSD1
1
2
MYB
me
me
H3K4
Promoter
H3K9
TSS
Gene
H3K9
body
ac